Controlling Hydrilla and Eurasian Watermilfoil with Fungal Pathogens from The People's Republic of China

PURPOSE: This technical note describes screening of fungal pathogens collected in The People's Republic of China for efficacy on hydrilla (*Hydrilla verticillata* (L.f.) Royle) and Eurasian watermilfoil or milfoil (*Myriophyllum spicatum* L.). Results of this research will be used to determine the direction of future efforts in using classical pathogen biological control research for management of these two nuisance aquatic weed species.

BACKGROUND: The principle of classical biocontrol is to search for natural enemies of a target plant in regions of the world where the plant and its enemies coevolved. Because hydrilla and Eurasian watermilfoil are widely distributed throughout the Old World, and their exact center of origin remains uncertain (Cook and Luond 1982; Couch and Nelson 1985), the search strategy to find natural enemies has been to survey specific areas in the reported range of the two species. Through support from the U.S. Army Engineer Aquatic Plant Control Research Program (APCRP), a classical pathogen biological control effort was launched in 1994. Survey work was initiated in Europe for pathogens of Eurasian watermilfoil (Harvey and Evans 1997) and in The People's Republic of China (PRC) (Shearer 1997a) for pathogens of both milfoil and hydrilla. Broadening the scope of the biological control program through foreign exploration for pathogens has the advantage of not only strengthening the existing pathogen program that currently uses only endemic organisms but enhancing the insect biocontrol program by increasing the number and variety of agents available for management of problematic submersed macrophytes.

With the easing of relations between the United States and the PRC in the late 1980's, a previously untapped region became accessible for exploratory research. Initiating surveys in the PRC offered the advantage of being able to simultaneously search for biological control agents for hydrilla and milfoil because both of these submersed macrophytes are indigenous to China. It also offered the opportunity to conduct surveys for agents in temperate areas where the climatic conditions more closely match areas in the United States where these plants have invaded.

The main objective of the research is to find classical biological control pathogens for hydrilla and Eurasian watermilfoil. The effort is divided into several phases that include foreign exploration, isolate identification, pathogenicity screening, efficacy evaluation on the target plants, and host specificity testing. The PRC exploration summaries and isolate identifications have been completed (Shearer 1997a). The third phase of the research reported herein was to screen potential pathogens for virulence on hydrilla and milfoil.

MATERIALS AND METHODS: During 1994 and 1995, pathogen surveys in the PRC resulted in the collection of over 200 fungal isolates from hydrilla and Eurasian watermilfoil tissues (Shearer 1997a). The isolates are deposited at the USDA-ARS Foreign Disease-Weed Science Laboratory (FDWSRU) located at Fort Detrick, Frederick, MD. As needed for screening evaluation, isolates were retrieved from cryostorage, thawed in 70-percent isopropyl alcohol for 15-20 min and plated

onto Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI) to initiate colony development. After 7 days, agar plugs were cut from the leading edge of each actively growing colony with a No. 4 cork borer. Three plugs were used to inoculate 250-ml Erlenmeyer flasks containing 150 ml of sterile Potato Dextrose Broth (PDB) (Difco Laboratories, Detroit, MI). A fourth plug was transferred to a one half strength Corn Meal Agar (CMA) slant (Difco Laboratories, Detroit, MI) to provide temporary storage of the isolate during the screening period. The flasks were agitated at 150 rpms on a rotary shaker for 6 days at room temperature.

Apical segments of hydrilla or milfoil cut to 15-cm lengths were placed in large test tubes (2.3 cm × 20 cm) containing 60 ml of sterile water. The fungal mycelial mat that developed during fermentation in the flasks was decanted into a sterile beaker and homogenized with a hand-held blender to a thick slurry. The slurry was inoculated onto the hydrilla and milfoil plants in 1-ml aliquots. Each treatment was replicated five times. The inoculated plants were placed in a 25 °C growth chamber (Conviron, Pembina, ND) set to a 14-hr photoperiod for 14 days.

The plants were evaluated for disease development at 7 and 14 days post inoculation. Each apical segment was assigned a disease rating based on a 0-4 scale: 0 = no macroscopic damage; 1 = slight chlorosis of leaves; 2 = general chlorosis of leaves and stems; 3 = general chlorosis and wilting of leaves and stems; 4 = total collapse or disruption of plant tissue. Isolates that induced a mean disease rating of 3 or 4 on the plants were retested using the methods described above.

In partial fulfillment of Koch's postulates, an attempt was made to reisolate the pathogens following disease evaluation. These reisolations were used to confirm that the organism retrieved from the diseased tissue matched the organism used for the inoculation. From each set of hydrilla and milfoil tubes, three pieces of plant tissue were collected at random, sterilized in a 0.5-percent NaOCL solution for 1 min and plated onto PDA amended with streptomycin sulfate.

RESULTS AND DISCUSSION: A total of 97 accessions of PRC isolates were screened for pathogenicity on hydrilla and Eurasian watermilfoil. Over half the isolates (66 of 97) induced some damage (i.e., a rating of '1' or greater) on the target plants. Several isolates caused damage to the plant species other than the source host, including 18 hydrilla isolates that damaged milfoil and 32 milfoil isolates that damaged hydrilla (Table 1).

Table 1
Evaluation of Selected Fungal Pathogens from the People's Republic of China for Disease-Producing Potential on Hydrilla and Milfoil Apical Stem Pieces

	Screen 1			Screen 2		
Isolate Source	Number of Isolates Evaluated	Milfoil[1]	Hydrilla[1]	Number of Isolates Evaluated	Milfoil[2]	Hydrilla[2]
Hydrilla	30	18	19	6	1	1
Milfoil	67	48	32	7	4	3
Total	97	66	51	13	5	4

[1] Isolates inducing a disease rating ≥ 1.
[2] Isolates inducing a disease rating ≥ 3.

During the first screening, 13 of the 97 isolates tested induced damage ratings of 3 or 4 on hydrilla or milfoil tissue and these were evaluated in a second screening. Of the six hydrilla isolates that were retested, only 1, *Phoma* sp., induced a level of disease that resulted in ratings that were comparable to the ratings in the initial evaluation (Table 2). The remaining five isolates induced chlorosis but no wilting or collapse of hydrilla plant tissue. It is not understood why the two isolates that caused hydrilla tissue collapse in the first test (i.e. a disease rating of 4) produced little or no damage in the second test. A third evaluation will be necessary before they are eliminated from consideration as biological control pathogens.

Table 2
Disease Rating Results from Screening Potential Biological Control Pathogens from the People's Republic of China on Hydrilla and Eurasian Watermilfoil

Isolate Number	Isolate Identification	Host Plant	Collection Site in PRC	Screen 1[1]		Screen 2[1]	
				Milfoil	Hydrilla	Milfoil	Hydrilla
C3294	Coelomycete 1	Hydrilla	Lan-dian-chang	2	4	1	0
C3394	Coelomycete 2	Hydrilla	San-jia-dian	1	4	1	1
C3694	Phoma sp.	Hydrilla	Qiao Zhuang	3	3	3	3
C4694	Colletotrichum gloeosporioides	Hydrilla	Han-jia-chuan	3	3	2	2
C6594	Mycoleptodiscus terrestris	Hydrilla	Huai-roi Reservoir	3	3	2	2
C6694	Mycoleptodiscus terrestris	Hydrilla	Huai-roi Reservoir	3	3	2	2
C5394	Pythium sp.[2]	Milfoil	San-jia-dian	3	2	--	--
C5594	Mycoleptodiscus terrestris[2]	Milfoil	Huai-roi Reservoir	2	3	--	--
C5594	Mycoleptodiscus terrestris	Milfoil	Huai-roi Reservoir	3	3	3	3
C5794	Mycoleptodiscus terrestris	Milfoil	Huai-roi Reservoir	3	3	3	3
C5894	Mycoleptodiscus terrestris	Milfoil	Huai-roi Reservoir	3	3	3	3
C5994	Cylindrocladium sp.	Milfoil	Huai-roi Reservoir	3	1	3	1
C2195	Coelomycete 2	Milfoil	East Lake, Hubei Province	2	3	2	2

[1] Disease rating scale: 0 = no macroscopic damage; 1 = slight chlorosis of leaves; 2 = general chlorosis of leaves and stems; 3 = general chlorosis and wilting of leaves and stems; 4 = total collapse or disruption of plant tissue.

[2] Isolates did not remain viable and were not tested in Screen 2.

Of the seven milfoil isolates that were retested, five produced comparable disease ratings on milfoil apical segments in the second test (Table 2). Three of the five isolates, all identified as *Mycoleptodiscus terrestris,* induced similar disease ratings on hydrilla tissue both in the initial screening and in the retest (Table 2). Two of the isolates could not be retested because they could not be induced to grow in culture in preparation for the second test.

M. terrestris is commonly isolated from hydrilla and milfoil plants collected throughout the United States and has been intensively investigated as a potential microbial biological control organism for management of hydrilla and milfoil (Shearer 1997b). The collection of the fungal pathogen from milfoil and hydrilla in the PRC was somewhat unexpected because surveying in the native range of a plant is undertaken with the intent of finding a new complex of natural enemies that are associated with the plant. *M. terrestris* is seemingly not present as a pathogen on milfoil in Europe because it was not isolated during the extensive survey work conducted on that continent during 1994 and 1995 (Harvey and Evans 1997). Because the pathogenicity of *M. terrestris* has been documented (Shearer 1997b), it was expected that some of the PRC isolates would also prove to be pathogenic on the intended hosts. In the initial test tube screening, six of the thirteen isolates that induced disease ratings of 3 or 4 on hydrilla and milfoil apical tissue were *M. terrestris*. In the second evaluation, three of the six *M. terrestris* isolates induced identical disease ratings (Table 2). Variability in disease-producing potential among United States isolates of *M. terrestris* has also been observed.[1] Currently, a strain from Texas has demonstrated the best potential for management of hydrilla and is undergoing evaluation and development as a bioherbicide (Shearer 1998). DNA analysis may help clarify if strains of the pathogen isolated from hydrilla and milfoil in the United States had their origin in Asia.

The two additional isolates that produced consistent results on hydrilla and milfoil tissue were *Phoma* sp. and *Cylindrocladium* sp. *Phoma* is a large genus with over 2,000 described species worldwide. Many of the species listed in *Fungi on Plant and Plant Products of the United States* (Farr et al. 1989) note only a single host, while others have a broad host range. They vary from highly pathogenic to merely saprophytic on the indicated hosts. Species within the genus *Cylindrocladium* also have a worldwide distribution and are described pathogens of a variety of angiosperm and gymnosperm host plants (Crous, Phillips, and Wingfield 1991). Most of the listed susceptible hosts are woody terrestrial plants. On the hosts, *Phoma* and *Cylindrocladium* spp. induce a variety of symptoms including leaf and stem lesions, seedling and shoot blight, root rot, damping off, and wilt. Several species of *Phoma* but no representatives from the genus *Cylindrocladium* have been isolated from milfoil and hydrilla in the United States. If additional work with the isolates indicates that either is a serious pathogen of milfoil, accurate identification to species will need to be undertaken.

FUTURE WORK: The small-scale screening tests indicate that five isolates from the PRC (3 *M. terrestris*, 1 *Phoma* sp., and 1 *Cylindrocladium* sp.) should undergo additional evaluation. The next phase of efficacy testing will challenge rooted hydrilla and milfoil plants in small columns or aquaria with mycelia or spores of the five isolates. If positive results are forthcoming, then dosage response and host specificity testing will be the next steps in the evaluation of the PRC isolates as classical biological control agents.

Foreign exploration for pathogens should be a top priority. To date, only a small area of the total native range of hydrilla and milfoil has been surveyed for pathogen biological control agents (Harvey and Evans 1997; Shearer 1997a). Plants in those regions were observed only during a short period of their growing season (i.e., each site was visited once). To adequately collect the maximum number of pathogens on the two target species, additional survey work is needed in other parts of

[1] Personal observation, 1999, Dr. Judy Shearer, Research Pathologist, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

the range of hydrilla and milfoil and sites should be revisited at different intervals during the growing season. Plants such as hydrilla and milfoil with native ranges that extend across broad land masses and climatic zones may have different complexes of organisms associated with them in different regions. Visiting sites at intervals during a growing season offers an opportunity to collect pathogens that infect seedlings, flowers, fruits, or mature plants. Such pathogens might easily be overlooked if surveys are not conducted during the appropriate growth cycle of the host.

POINTS OF CONTACT: For additional information, contact the author, Dr. Judy F. Shearer, (601) 634-2516, *shearej@wes.army.mil* or the managers of the Aquatic Plant Control Research Program, Dr. John W. Barko, (601) 634-3654, *barkoj@wes.army.mil* and Mr. Robert C. Gunkel, Jr., (601) 634-3722, *gunkelr@wes.army.mil*. This technical note should be cited as follows:

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REFERENCES

Cook, C. D. K., and Luond, R. (1982). "A revision of the genus *Hydrilla* (Hydrocharitaceae)," *Aquat Bot.* 13,485-504.

Couch, R., and Nelson, E. (1985). "*Myriophyllum spicatum* in North America." *Proceedings of the First International Symposium on watermilfoil (Myriophyllum spicatum) and related Haloragaceae species*. L.W.J. Anderson, ed., Aquat. Plant Manage. Soc., Washington, DC, 8-18.

Crous, P. W., Phillips, A. J. L., and Wingfield, M. J. (1991). "The general *Cylindrocladium* and *Cylindrocladiella* in South Africa, with special reference to forest nurseries," *South African Forestry Journal* 157,69-85.

Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. (1989). *Fungi on plants and plant products in the United States*. APS Press, St. Paul, MN.

Harvey, J. L., and Evans, H. C. (1997). "Assessment of fungal pathogens as biocontrol agents of *Myriophyllum spicatum*," Miscellaneous Paper A-97-3, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J. F. (1997a). "Classical pathogen biocontrol research in Asia 1994-1995: Surveys for pathogen agents of *Hydrilla verticillata* (L.f.) Royle and *Myriophyllum spicatum* L," Technical Report A-97-1, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J. F. (1997b). "Endemic pathogen biocontrol research on submersed macrophytes: Status Report 1996," Technical Report A-97-3, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J. F. (1998). "Biological control of hydrilla using an endemic fungal pathogen," *J. Aquat. Plant Management* 36:54-56.